Role of Girdin in intimal hyperplasia in vein grafts and efficacy of atelocollagen-mediated application of small interfering RNA for vein graft failure

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Objective: **Intimal hyperplasia is a major obstacle to patency in grafted veins. Although migration and proliferation of vascular smooth muscle cells (SMCs) pivotally affect the vascular remodeling process, no therapy has been established to prevent intimal hyperplasia of vein grafts. We previously reported that the actin-binding protein Girdin crucially affects arterial remodeling. In this study, we investigated the role of Girdin in venous SMCs and evaluated a therapeutic strategy for vein graft failure in vivo using small interfering RNA (siRNA) that targets *Girdin*.**

Methods: **We investigated the relationship between Girdin expression and intimal hyperplasia using a rabbit vein graft model. Vein grafts under low-flow conditions were performed in Japanese White rabbits. For in vitro analyses, we isolated primary venous SMCs from vein graft neointima. siRNA that targets *Girdin* was mixed with atelocollagen, which stabilizes and releases nucleic acid reagents slowly and is applied perivascularly to the vein grafts at operation. Intimal hyperplasia was evaluated 4 weeks later.**

Results: **In the rabbit model, increased Girdin expression was seen in the neointima after the grafting operation. Using primary venous SMCs, we showed that Girdin is required for rearrangement of the actin cytoskeleton in venous SMCs and that siRNA-mediated *Girdin* knockdown significantly reduced venous SMC migration and proliferation. *Girdin* knockdown via perivascular application of siRNA using atelocollagen markedly reduced intimal thickening after the grafting operation.**

Conclusions: **Depletion of Girdin attenuated venous SMCs migration and proliferation in vitro and intimal hyperplasia in vein grafts in vivo. Our findings suggest that Girdin affects migration and proliferation of vascular SMCs in vein grafts and that controlled release of *Girdin* siRNA using atelocollagen could be a novel therapeutic strategy for vein graft failure. (J Vasc Surg 2014;60:479-89.)**

Clinical Relevance: **Intimal hyperplasia is a major obstacle to patency after vein grafting. Although migration and proliferation of vascular smooth muscle cells pivotally affect the vascular remodeling process, no therapy has been established to prevent intimal hyperplasia of vein grafts. Here, we report that depletion of the actin-binding protein Girdin attenuated migration and proliferation of venous smooth muscle cells in vitro and intimal hyperplasia in vein grafts in vivo. Our findings indicate that perivascular application of *Girdin* small interfering RNA using atelocollagen could be a novel therapeutic strategy for vein graft failure.**

Cardiovascular disease is a major cause of death and disability in developed countries. More than 2200 Americans die of cardiovascular disease each day, an average of one death every 39 seconds.[1] An autologous vein is the most commonly used conduit for coronary and peripheral artery bypass grafting in treating ischemia resulting from occlusive vascular disease. However, the long-term success of this operation is limited by vein graft occlusion caused by intimal hyperplasia and superimposed atherosclerosis, with worse patency for poor runoff vessels in which hemodynamic factors, such as low-flow velocity and low shear stress, cause intimal thickening.[2,3]

Increased proliferation of terminally differentiated vascular smooth muscle cells (SMCs) contributes significantly to lesional neointima formation.[4] Neointimal SMCs are controversially reported to originate from local vessel walls, circulating progenitor cells, or from bone marrow.[5-9] Despite many clinical trials, few models reflect the contribution of various cell types to neointimal development, nor has a satisfactory therapeutic strategy been established.

The phosphatidylinositol 3-kinase (PI3K)/Akt system regulates multiple cellular processes through phosphorylation of downstream substrates. The PI3K/Akt pathway and its downstream components are also pivotal in vascular remodeling.[10] Girdin (girders of actin filaments), also known as Gα-interacting vesicle-associated protein, is

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a novel actin-binding Akt substrate.[11-14] Girdin is expressed at high levels in cell types that require remodeling of actin filaments, cell migration, and proliferation such as SMCs, neuroblasts, and cancer cells.[11,15-19]

We reported that Girdin affects migration and proliferation of arterial SMCs and regulates neointima formation after arterial injury via Akt-mediated phosphorylation.[18] However, little information is available about its functions in venous SMCs, especially in vein grafts. Here, we studied the roles of Girdin in migration and proliferation of venous SMCs in vitro and found a combination of small interfering RNA (siRNA) that targets *Girdin* and atelocollagen, which allows its controlled release, to suppress significantly intimal hyperplasia in vein grafts in vivo. These findings provide a novel strategy to prevent intimal hyperplasia.

METHODS

The animal experiments in this study were performed in compliance with the guidelines of Nagoya University Graduate School of Medicine and approved by the Animal Care and Use Committee (Permit Number: 24084).

Antibodies. Rabbit anti-Girdin polyclonal antibody was developed in-house against the 19 carboxyl-terminal amino acids of Girdin and affinity-purified with the immunizing peptide.[11] Other primary antibodies used in this study were commercial sheep polyclonal anti-Girdin (R&D Biosystems, Minneapolis, Minn), mouse monoclonal anti-α-smooth-muscle actin (α-SMA) and anti-Ki-67 antibodies (Dako, Glostrup, Denmark), mouse monoclonal anti-β-actin antibody (Sigma, St. Louis, Mo), and rat monoclonal anti-CD31 antibody (Dianova, Hamburg, Germany).

Vein graft model. Japanese White rabbits (2.5-3.0 kg) were anesthetized by an intramuscular administration of ketamine (35 mg/kg) and xylazine (10 mg/kg). The no-touch technique was used to dissect a segment of right external jugular vein (20-mm length; 3-mm width) through a midline vertical neck incision. All branches were carefully ligated with 8-0 polypropylene sutures and divided. Animals were systemically heparinized (200 U/kg). The ipsilateral common carotid artery was clamped distally and proximally, and a graft was anastomosed in end-to-end fashion into the divided artery with interrupted 8-0 polypropylene sutures.

Before wound closure, grafts were subjected to low-flow conditions, as described previously (poor runoff model).[20,21] The poor runoff model mimics clinical conditions of vein grafts performed for ischemic extremities with poor runoff. For poor runoff models, three of four distal vessel branches were ligated after the external jugular vein-to-carotid artery interposition (Fig 1, *A*).[2,3] The distal vessels remained patent in the normal runoff model.

Primary culture of venous SMCs. The experiments were performed with primary cultures of venous SMCs from rabbit vein grafts 4 weeks after bypass grafting. The entire length of the vein grafts was immediately excised under sterile conditions. The vein grafts were rinsed with Hanks solution and opened lengthways. The neointima was dissected from the underlying media. The separated sheets of neointima were cut into small pieces of ~1 mm^2, washed in Hanks solution, and incubated in Hanks solution containing collagenase I (1 g/L; Funakoshi, Tokyo, Japan) and elastase III (0.5 g/L; Sigma) for 90 minutes at 37°C.

Thereafter, the cells were dispersed. The cell suspension was centrifuged at 120 *g* for 4 minutes, resuspended, and grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 1% streptomycin/penicillin, and 2 mM L-glutamine. The identity of SMCs was confirmed by immunostaining with anti-α-SMA antibody. Venous SMCs at passages one to three were used in the experiments.

RNA interference. siRNA-mediated knockdown of Girdin was performed using "siRNA (B)" in vitro, as described previously.[11,18] In addition, we made two more siRNAs for rabbit Girdin, as follows (sense sequence only):

si-rb1: 5′-GGACCAACCUGGAUGAAUATT-3′ (nucleotides 98-116), and

si-rb2: 5′-GGCAGAACAUCCACUAGCATT-3′ (nucleotides 4768-4786).

The 21-nucleotide synthetic duplexes with two 3′-end overhang dT were prepared by Qiagen (Valencia, Calif).

Venous SMCs were transfected with one of the siRNAs (50 nM) or a 21-nucleotide irrelevant RNA (Qiagen) as a control, using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, Calif), according to the manufacturer's protocol. siRNAs were successfully introduced into almost 100% of SMCs using Lipofectamine RNAiMAX.

For the in vivo study, we used stabilized siRNAs, Dharmacon siSTABLE (Thermo Scientific, Lafayette, Colo). We used atelocollagen-based in vivo siRNA/microRNA transfection reagent AteloGene Local Use (Koken Co Ltd, Tokyo, Japan). AteloGene is a highly purified type I collagen derived from calf dermis by pepsin treatment. Before the operation, siRNA-AteloGene complexes were prepared as follows: 150 μL of AteloGene and 150 μL of a stabilized siRNA solution (20 μM) were mixed, maintained at 4°C, and then used to coat the external surface of the vein graft. This complex formed a gel after appropriate heat treatment with a hair dryer for <10 seconds, which did not dry or injure the vein grafts.[20] This siRNA-containing gel remained around the graft for at least 1 week.

Western blot analysis. Frozen rabbit vein grafts were homogenized in radioimmunoprecipitation assay buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% nonyl phenoxypolyethoxylethanol, 0.1% sodium dodecyl sulfate, and 0.1% sodium deoxycholate and rotated at 4 rpm for 2 hours at 4°C. After protein concentrations were measured, lysates were boiled at 100°C for 2 minutes in the presence of 2% β-mercaptoethanol. The lysates, containing 30 μg of proteins, were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blotting was performed using standard protocols detailed in the Supplementary Methods (online only).

Histopathology. Intact veins and vein grafts were fixed at 100 mm Hg infusion pressure for 20 minutes with 10% neutral-buffered formalin and immersed for 40 hours in 10% neutral-buffered formalin. Three evenly spaced segments (4-5 mm apart) of the middle portion of each graft

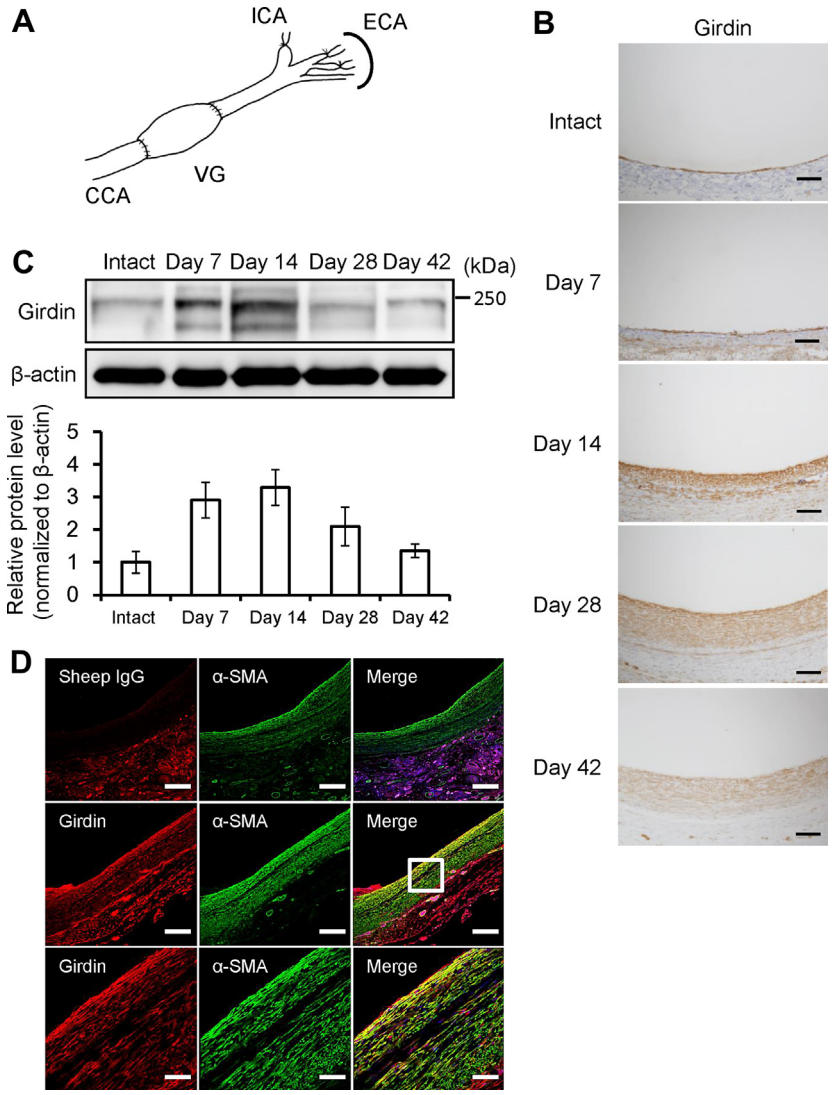

Fig 1. Girdin localizes to vascular smooth muscle cells (SMCs) in the tunica media and neointima of vein grafts. **A,** Poor runoff vein graft (*VG*) model with distal branches ligation is depicted. The inferior-most branch of the external carotid artery (*ECA*) is the only outflow for the low-flow graft. *CCA*, Common carotid artery; *ICA*, internal carotid artery. **B,** Rabbit vein grafts at the indicated days postoperation underwent immunohistochemistry analysis using anti-Girdin antibody. Note that these veins and vein grafts were dilated with infusion pressure during fixation, and vascular walls look thinner than under normal conditions. The *scale bar* = 100 μm. **C,** Western blot analyses show Girdin expression in rabbit whole-vein grafts. The bar graph shows protein levels of Girdin (relative to β-actin) determined by densitometry in whole-vein grafts harvested at the indicated days postoperation. The Girdin level in the intact vein was defined as 1. The *error bars* indicate the standard error of the mean (n = 3 for each group). * $P < .05$. **D,** Vein grafts at 14 days postoperation underwent immunofluorescence staining using sheep immunoglobulin G (*IgG*) as a negative control or anti-Girdin antibody (*red*) and anti-α-smooth muscle actin (*SMA*) antibody (*green*). Cell nuclei were labeled with 4′,6-diamidino-2-phenylindole. **Upper** and **middle panels,** Representative photomicrographs are at low magnifications (*scale bars* = 200 μm). **Lower panel,** The *boxed area* is magnified (*scale bars* = 50 μm).

were then embedded in paraffin. Serial sections were stained with hematoxylin and eosin or elastica van Gieson. Intimal and medial cross-sectional areas were measured, and the neointimal thickness was measured at eight randomly selected points for each section using MetaMorph software (Universal Imaging, Ypsilanti, Mich).

Immunohistochemical analysis. Sections were heated in Target Retrieval Solution (pH 9.0; Dako) for antigen retrieval, blocked with Protein Block Serum-Free Ready-to-Use (Dako), incubated with primary antibodies, washed in phosphate-buffered saline, and incubated with the secondary antibody. Reaction products were visualized

with diaminobenzidine (Dako); nuclear counterstaining used hematoxylin.

Immunofluorescence staining. Immunofluorescence staining was performed as previously described[11]; protocols are detailed in the Supplementary Methods (online only).

Quantification of lamellipodia. We investigated the increased number of cells with extended lamellipodia; that is, the difference in the number of cells with extended lamellipodia between before and after platelet-derived growth factor (PDGF) stimulation. Note that cells with lamellipodia at more than one-third (>120°) of the cell periphery were counted as cells with extended lamellipodia.

Cell migration and mitogenic activity assays. Directional cell migration of venous SMCs was stimulated in a monolayer using an in vitro scratch wound assay, as previously described.[15] Mitogenic activity was measured using the Cell Proliferation Reagent WST-1 assay (Roche Applied Science, Penzberg, Germany). Protocols for these assays are detailed in the Supplementary Methods (online only).

Detection of cell proliferation and apoptosis in vivo. Nuclei positive for Ki-67 or terminal deoxynucleotide transferase-mediated deoxy uridine triphosphate nick-end labeling (TUNEL) were evaluated, as previously described.[18,20] To detect proliferation, sections of rabbit vein grafts were blocked and incubated with a mouse monoclonal anti-Ki-67 antibody overnight at 4°C. Thereafter, sections were incubated with EnVision+ System-HRP Labelled Polymer Anti-mouse (Dako). To detect apoptosis, we used the Apoptosis in situ Detection Kit (Wako, Osaka, Japan), according to the manufacturer's protocol. In each experiment, hematoxylin was used for nuclear counterstaining. Numbers of Ki-67$^+$ and TUNEL$^+$ cells were calculated as Ki-67 or TUNEL indices (Ki-67$^+$ or TUNEL$^+$ cells/total cells × 100), respectively.

Statistical analysis. Values are expressed as means ± standard error of the means. Significance ($P < .05$) was determined using the Student *t*-test or Bonferroni post hoc analysis.

RESULTS

Increased expression of Girdin in neointima of vein grafts. To examine the effects of Girdin in vein grafts, Girdin expression in cross-sections prepared from rabbit vein grafts with poor runoff was assessed immunohistochemically. Girdin expression was detected in the media of the intact vein that was α-SMA$^+$ (Fig 1, *B*; Supplementary Fig 1, online only). As the neointima thickened, enhanced Girdin expression was observed in the neointima rather than in the media (Fig 1, *B*). Girdin expression in the neointima peaked 14 days after bypass grafting and gradually decreased thereafter.

Girdin expression in whole intact veins and vein grafts with poor runoff was studied using Western blot analyses (Fig 1, *C*). Only weak Girdin expression was detected in intact veins. Expression levels increased after vein grafting and peaked at day 14 after bypass grafting, which was consistent with immunohistochemical analysis. Girdin localization was confirmed in 14-day-old vein grafts by immunofluorescence staining using anti-Girdin antibody and anti-α-SMA antibody (Fig 1, *D*). Girdin localizes to α-SMA$^+$ cells in the media and neointima. Cells that were both Girdin$^+$ and α-SMA$^+$ existed mainly in the vein graft neointima at 14 days after bypass grafting. This finding suggests that Girdin is upregulated in the developing neointima.

Girdin is upregulated in the vein grafts with poor runoff. To determine whether Girdin affects intimal hyperplasia in vein grafts, we compared Girdin expression in vein grafts with poor and normal runoff. The neointima was clearly thickened in vein grafts with poor runoff compared with normal runoff models (Fig 2, *A*), consistent with previously reported data.[3] All vein grafts of both models were patent when they were excised. Western blot analyses showed that Girdin expression markedly increased during neointima formation at 7 and 14 days after bypass grafting in poor runoff models compared with normal runoff models (Fig 2, *B*).

Rearrangement of actin cytoskeleton of venous SMCs requires Girdin. We examined Girdin localization using primary venous SMCs isolated from the neointima of vein grafts. Immunocytochemical analysis using anti-Girdin and anti-β-actin antibodies showed Girdin localized on actin stress fibers in venous SMCs (Fig 3, *A*), as previously observed in other cell types.[15,18] To investigate Girdin function in venous SMCs, we observed the effect of RNA-mediated interference on actin cytoskeletons in these cells. We introduced three siRNAs targeting *Girdin* (siRNA [B], si-rb1, si-rb2) and an irrelevant siRNA (control siRNA) into venous SMCs. Although serum-starved controls and Girdin-depleted venous SMCs showed no apparent morphologic differences, formation of thick stress fibers significantly decreased in Girdin-depleted cells stimulated with serum compared with control cells (Fig 3, *B*). Moreover, after stimulation with PDGF-BB, the leading-edge extension of lamellipodia was significantly attenuated, and only small membrane protrusions could be seen in the Girdin-depleted cells (Fig 3, *C*). The increased number of Girdin-depleted cells with extended (>120°) lamellipodia after PDGF stimulation was significantly lower than that of control cells, at 14.49 ± 3.19 and 1.95 ± 1.57 per 100 cells, respectively ($n = 3$; $P < .05$; Fig 3, *D*). These findings suggest that Girdin pivotally affects actin remodeling of lamellipodia in migrating venous SMCs.

Girdin depletion inhibits venous SMCs migration and proliferation in vitro. Vascular SMCs migration and proliferation are reported to be key events in intimal hyperplasia of vein grafts.[4] We investigated whether Girdin depletion inhibits venous SMCs migration in vitro using wound healing assays (Fig 4, *A*). siRNA-mediated *Girdin* knockdown resulted in significantly reduced migration distance by venous SMCs and fewer cells migrating into the wounded area, compared with those of venous SMCs transfected with control siRNA ($n = 3$; $P < .01$; Fig 4, *B*

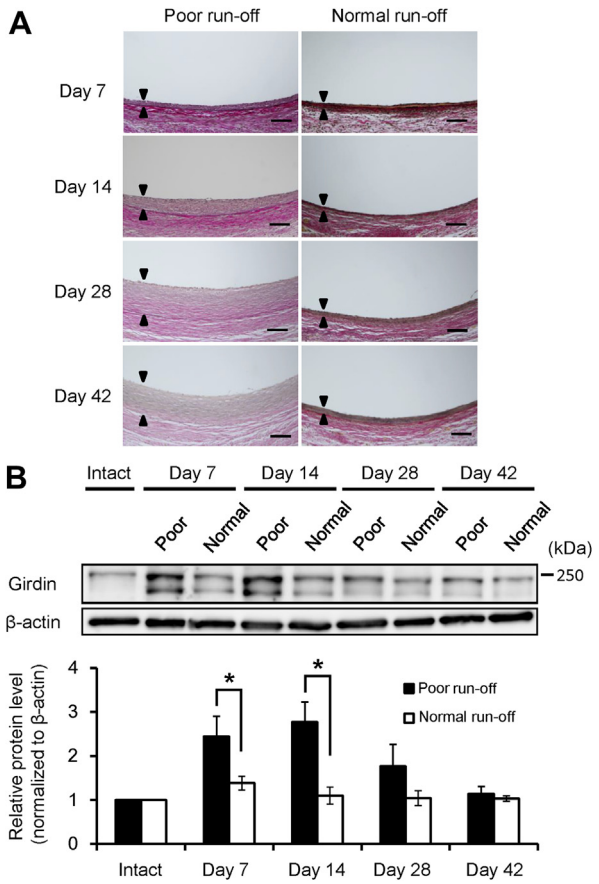

Fig 2. Girdin is upregulated in vein grafts with poor runoff. **A,** Representative elastica van Gieson-stained sections of vein grafts harvested at indicated days postoperation. The *lower arrowheads* indicate the internal elastic lamina. The *scale bar* = 100 μm. **B,** Western blot analyses showed Girdin expression in rabbit whole-vein grafts with poor or normal runoff. The bar graph shows Girdin protein levels (relative to β-actin) determined by densitometry in whole-vein grafts harvested at the indicated days postoperation. The Girdin level in intact veins was defined as 1. The *error bars* show the standard error of the mean (n = 3 for each group). **P* < .05.

and *C*). Next, we used water-soluble tetrazolium salt (WST)-1 assays to examine the effect of Girdin on venous SMC proliferation. On day 4, proliferation of Girdin-depleted cells was significantly suppressed compared with control cells (n = 5; *P* < .01; Fig 4, *D*). These findings show that Girdin depletion inhibits migration and proliferation of venous SMCs in vitro, suggesting that use of siRNA targeting *Girdin* could be a novel treatment for intimal hyperplasia of vein grafts.

Girdin depletion inhibits intimal hyperplasia in vein grafts in vivo. Because the siRNAs targeting *Girdin* (siRNA [B], si-rb1, si-rb2) had similar biological effects in vitro (Figs 3 and 4, data not shown) and transfection with si-rb2 tended to more strongly suppress Girdin expression (Supplementary Fig 2, *A*, online only), we adopted the sequence of si-rb2 for the in vivo study. After initially confirming that stabilized siRNA (siSTABLE) retained inhibitory effects equal to those of unmodified siRNA (Supplementary Fig 2, *B*, online only), we used siSTABLE for all animal experiments. Vein grafts were treated with *Girdin* siRNA, as described in the Methods section. We applied *Girdin* siRNA mixed with atelocollagen perivascularly to vein grafts at operation (Supplementary Fig 3, *A*, online only).

Western blot analysis confirmed that *Girdin* siRNA effectively suppressed Girdin expression in vein grafts in vivo at least for 14 days postoperation (Fig 5, *A*); we therefore evaluated its potential to inhibit intimal hyperplasia. At 4 weeks postoperation, the intimal thickness and the intima-to-media ratio were 0.09 ± 0.01 mm and 0.67 ± 0.08, respectively, for vein grafts treated with atelocollagen-mediated local application of *Girdin* siRNA (n = 5) and 0.22 ± 0.05 mm and 1.76 ± 0.24, respectively, for grafts treated with atelocollagen containing irrelevant siRNA (n = 5; Fig 5, *B-D*).

In addition, intimal thickness and the intima-to-media ratio did not significantly differ between vein grafts treated with atelocollagen containing irrelevant siRNA and non-treated grafts, indicating that controlled release of *Girdin* siRNA using atelocollagen reduced intimal hyperplasia of rabbit vein grafts significantly (*P* < .05), whereas atelocollagen alone had no effect. However, medial thickness did not differ among the three groups at 4 weeks postoperation (Supplementary Fig 3, *B*, online only).

Increased endothelial coverage reportedly correlates with diminished intimal hyperplasia.[22] To further investigate the effects of *Girdin* knockdown in vein grafts, we evaluated re-endothelialization in vein grafts 7 days after bypass grafting. Endothelial coverage in vein grafts was assessed immunohistochemically using anti-CD31 antibody (Supplementary Fig 4, *A*, online only). Quantification with image analysis showed no significant difference in endothelial coverage between *Girdin* siRNA-treated vein grafts and control siRNA-treated grafts (Supplementary Fig 4, *B*, online only). This suggests that the inhibitory effect of *Girdin* knockdown on intimal hyperplasia was unrelated to re-endothelialization.

Girdin is involved in cell proliferation, but not cell survival, in vein grafts. Cell migration, cell proliferation, and apoptosis of vascular SMCs are major steps in intimal hyperplasia.[23,24] To examine cell proliferation and apoptosis in vein grafts, we performed Ki-67 immunostaining and TUNEL staining, respectively (Fig 6, *A* and *D*). Vein grafts treated with *Girdin* siRNA showed significantly fewer Ki-67$^+$ proliferating cells from media to neointima at 7 days postoperation compared with vein grafts treated with control siRNA (Fig 6, *B*). The media and neointima were difficult to distinguish at 7 days postoperation. At 28 days postoperation, Ki-67$^+$ cells significantly increased in the neointima but not in the media (Fig 6, *C*). Conversely, cell survival in neither neointima nor media was affected significantly in vein grafts treated with *Girdin* siRNA at any point (Fig 6, *E* and *F*). These

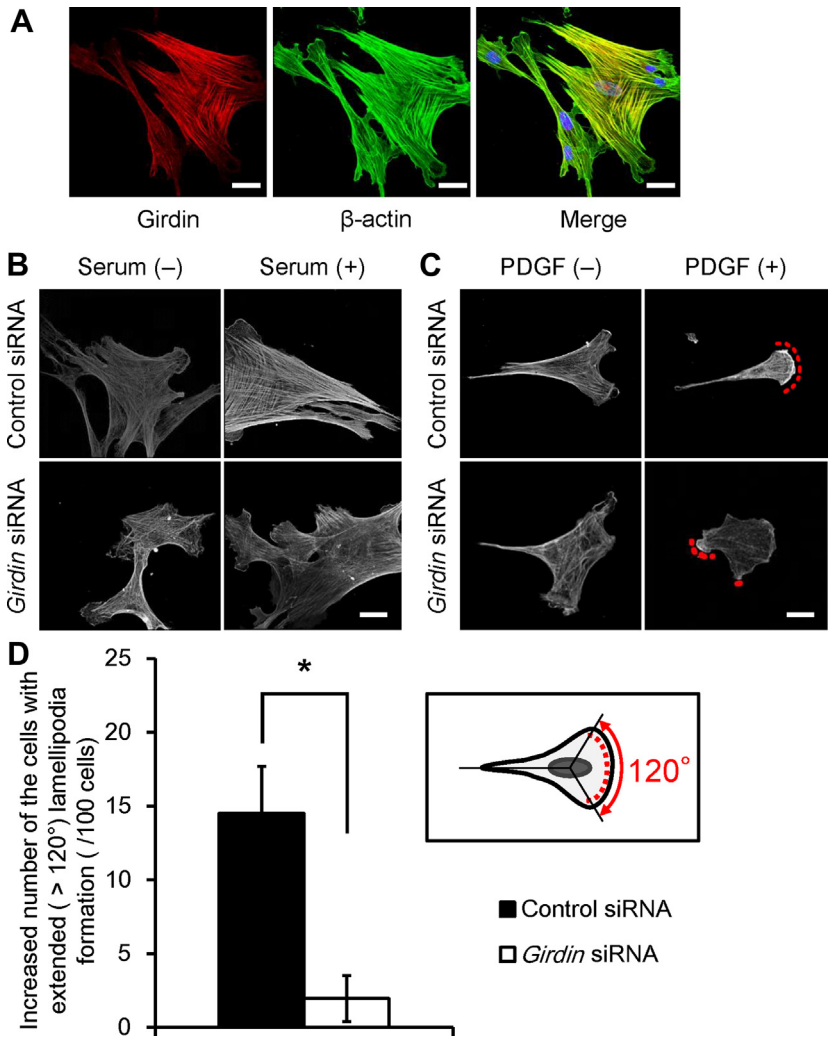

Fig 3. Girdin is required to rearrange actin cytoskeletons in venous smooth muscle cells (SMCs). **A,** Venous SMCs were stained using anti-Girdin antibody and anti-β-actin antibody. Girdin localizes to actin stress fibers. The *scale bar* = 20 μm. **B,** Venous SMCs transfected with control or *Girdin* small interfering RNA (*siRNA*; si-rb2) were stimulated with serum to induce stress fiber formation, which was immunostained using anti-β-actin antibody. The *scale bar* = 20 μm. **C,** Venous SMCs were stimulated with platelet-derived growth factor (*PDGF*)-BB (20 ng/mL) for 10 minutes and stained with anti-β-actin antibody. The *red dotted lines* denote lamellipodia at the leading edge. The *scale bar* = 20 μm. **D,** In experiments described in **C,** the increased number of cells with extended lamellipodia formation after PDGF stimulation was quantified. The *error bars* show the standard error of the mean (n = 3 for each group). **P* < .05. The *inset* shows a schematic illustration of extended lamellipodia formation. Cells with lamellipodia at more than one-third (>120°) of the cell periphery were counted as cells with extended lamellipodia.

results suggest that in vein grafts, Girdin affects cell proliferation but not cell survival.

DISCUSSION

Because we previously reported that Girdin depletion reduces migration and proliferation of arterial SMCs,[18] we hypothesized that *Girdin* knockdown would retard intimal hyperplasia in vein grafts. Immunohistochemical analyses confirmed that Girdin localized to vascular SMCs in the media and neointima of rabbit vein grafts. Girdin expression in the neointima peaked at ~14 days after implantation. These changes in Girdin expression in vein grafts resembled those of vascular SMC proliferation in vein grafts.[25] In poor runoff models with abnormal blood flow conditions, as characterized by low flow and low shear stress variation mimicking clinical conditions of vein grafts performed for ischemic extremities, intimal graft thickening is more progressive than that in control models with normal runoff.[2,3] We showed that Girdin expression in vein grafts with poor runoff was greater than that in

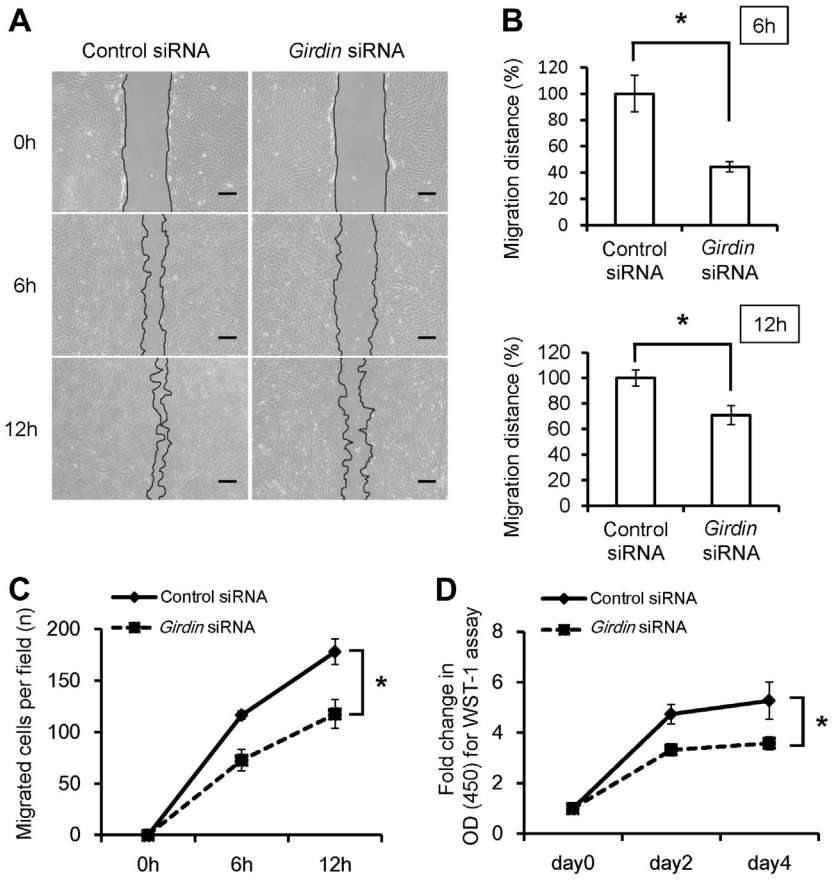

Fig 4. Depletion of Girdin inhibits venous smooth muscle cell (SMC) migration and proliferation in vitro. **A,** Venous SMCs transfected with control or *Girdin* small interfering RNA (*siRNA*; si-rb2) were cultured in growth medium containing 10% fetal bovine serum. Cells were allowed to migrate toward the wound for 12 hours. The *scale bar* = 300 μm. **B,** Ratio of the migration distance in each group at each time (n = 6 in each group). **C,** Number of cells that had migrated into the wounded area (n = 6 in each group). Three independent experiments were performed. The *error bars* show the standard error of the mean. **P* < .05. **D,** A water-soluble tetrazolium salt (WST)-1 assay was performed to assess cell proliferation. Absorbance of each cell at day 0 was defined as 1. The *error bars* show the standard error of the mean (n = 5). **P* < .05. *OD,* Optical density.

vein grafts with normal runoff. These results suggest that Girdin contributes to vascular SMC proliferation in vein grafts and intimal hyperplasia after bypass grafting.

We isolated primary venous SMCs from enzyme-dispersed neointima of rabbit vein grafts 4 weeks postoperation. These primary cells, apparently originating from various cell types, exhibited increased migration and proliferation shortly after being isolated. In contrast, cells isolated from vein grafts 8 weeks postoperation in the same way did not migrate readily and rarely underwent mitosis in the first few days (data not shown). In this study, we isolated primary venous SMCs from the neointima of vein grafts by the enzyme dispersion method, although isolation of venous SMCs by the explant method has recently been reported.[26] We suppose that cells prepared in this way reflect the form of vascular SMCs in the vein graft more exactly than those isolated from explants of vena cava or saphenous veins, because cell types from neointimal SMCs are different from those of medial SMCs,[27] and the enzyme dispersion method is known to retain characteristic features of in vivo cells.[28] Our data showed that Girdin localized to venous SMCs, especially in the neointima of vein grafts 14 days after implantation, rather than those in the media.

Plausibly, Girdin might be expressed in dedifferentiated vascular SMCs and affect phenotypic modulation in vascular SMCs because vein graft neointima have abundant dedifferentiated vascular SMCs.[21] To examine this hypothesis, we stimulated venous SMCs with PDGF-BB (a known inducer of phenotypic switch in vascular SMCs) containing 10% fetal bovine serum, which markedly enhanced Girdin expression (Supplementary Fig 5, *A*, online only) and led to morphologic change (Supplementary Fig 5, *B*, online only). Although our data suggest that Girdin is upregulated in dedifferentiated vascular SMCs, further studies are needed to understand the role of Girdin in vascular SMC phenotypic modulation.

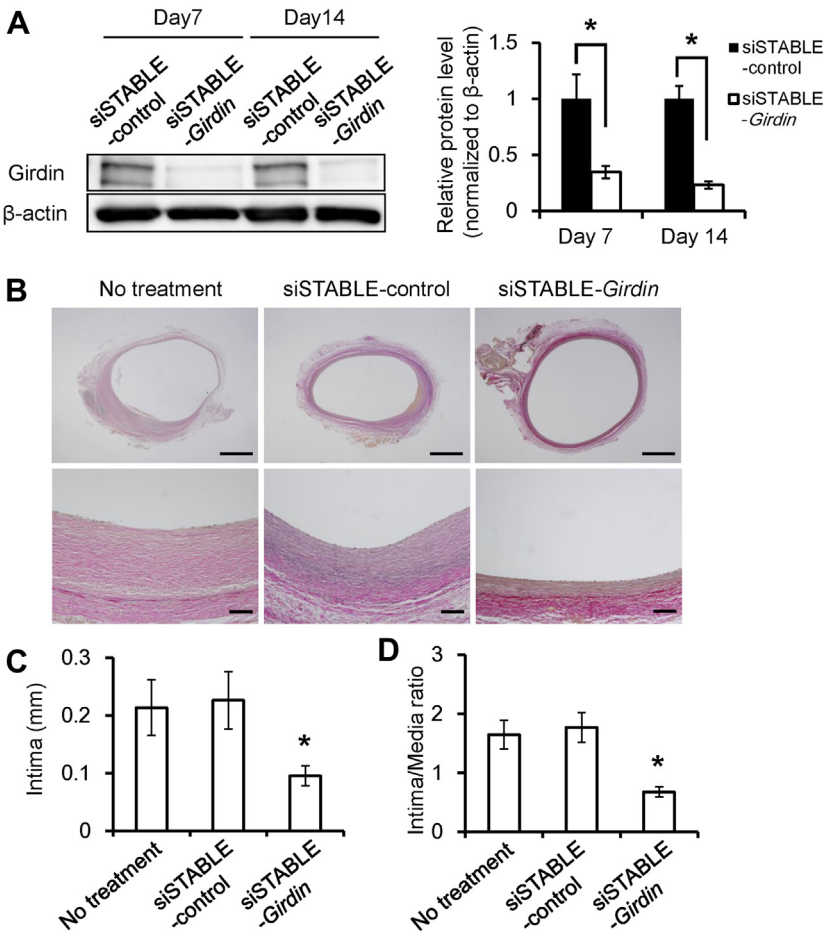

Fig 5. Depletion of Girdin inhibits intimal hyperplasia in vein grafts in vivo. **A,** Western blot analyses show efficacy of the treatment with atelocollagen containing *Girdin* small interfering RNA (siRNA) in whole-vein grafts at 7 and 14 days postoperation. The bar graph shows relative protein levels of Girdin determined by densitometry. The *error bars* show the standard error of the mean (n = 3). **P* < .05. **B,** Representative elastica Van Gieson-stained sections from animals at 4 weeks postoperation, with or without *Girdin* siRNA treatment. Representative photographs at **(upper panel)** low (*scale bar* = 1.0 mm) and **(lower panel)** high (*scale bar* = 100 μm) magnifications are presented. Significant reduction was seen in **(C)** intimal thickness and the **(D)** intima/media ratio in vein grafts treated with *Girdin* siRNA at 4 weeks postoperation. The *error bars* indicate the standard error of the mean (n = 5 for each group). **P* < .05.

Despite intense research, the optimal vector for gene delivery to the vasculature remains somewhat elusive. Such a vector should efficiently transduce target vascular cells with minimal transduction of nontarget cells, have low toxicity and immunogenicity, and be sufficiently stable to allow longevity of transgene expression, leading to an adequate clinical response.[29] Although the most commonly used vector is an adenovirus, viral vectors pose three main limitations in human vascular gene therapy: (1) common pre-existing immunity to the adenovirus, (2) profound immune response generated to adenoviral-transduced cells, and (3) direct tissue toxicity.[30] Many trials, including genetic interventions to prevent vein graft failure, take advantage of the fact that veins to be grafted can be manipulated ex vivo. Antisense or decoy oligodeoxynucleotides have been transfected into vein graft walls by using liposomes or high pressure.[31-35] Newer methods, such as ultrasound or gene guns, deliver desired genes by nonviral vectors; for example, plasmids through cellular walls.[36] This strategy, however, allows a drug to be administered only once, and most of the applied drug remains at the application site relatively briefly. Because neointimal SMCs have three potential sources—vein grafts, arterial walls, and circulating blood—single treatments for vein grafts are probably insufficient.[7]

Here, we used atelocollagen to deliver siRNA. Atelocollagen is a liquid at 4°C and becomes solid at 37°C. It has been used as a delivery system for expression plasmids, antisense oligodeoxynucleotides, and siRNAs because it stabilizes and slowly releases nucleic acid reagents.[37-39] Thus, siRNA is retained at the application site, allowing long-lasting target gene suppression in vascular SMCs without

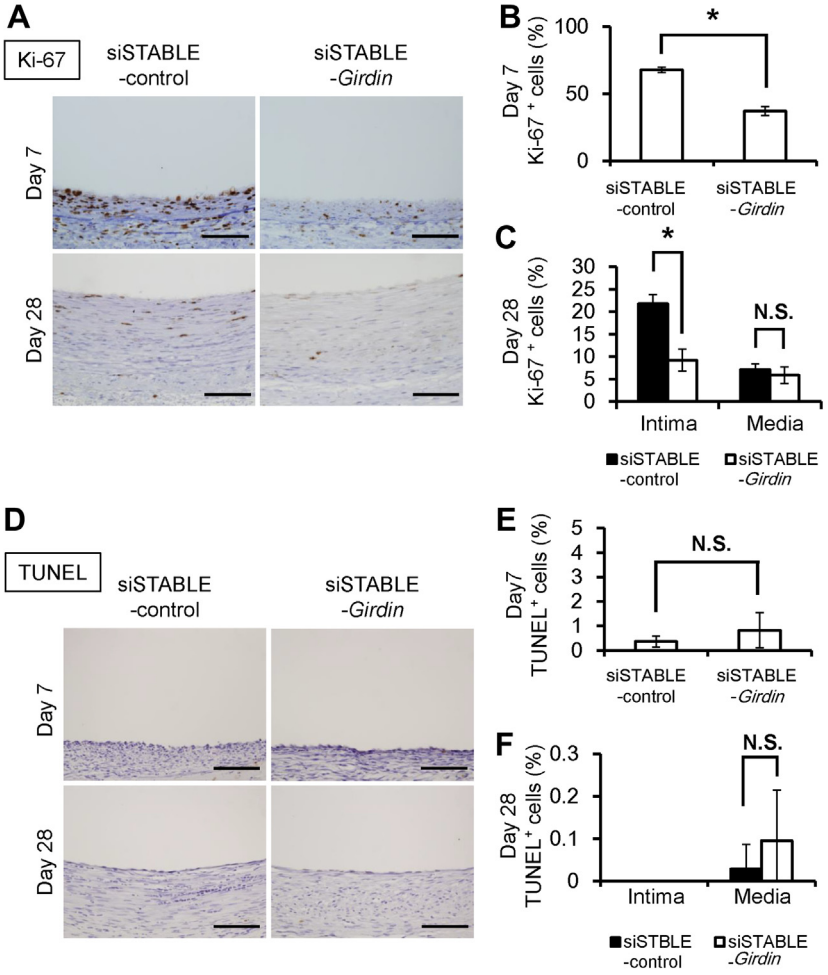

Fig 6. Cell proliferation and survival in vein grafts postoperation. **A,** Ki-67-immunostained sections of vein grafts treated with *Girdin* small interfering RNA (siRNA) or control siRNA. The *scale bar* = 100 μm. **B,** Percentage of Ki-67$^+$ cells in area inside of vein graft internal elastic lamina 7 days postoperation. **C,** Percentage of Ki-67$^+$ cells in the media or intima of vein grafts 28 days postoperation. The *error bars* indicate the standard error of the mean (day 7, n = 3; day 28, n = 5). *$P < .05$. *N.S.,* Not significant. **D,** Vein grafts underwent terminal deoxynucleotide transferase-mediated deoxy uridine triphosphate nick-end labeling (*TUNEL*) staining at 7 or 28 days postoperation. The *scale bar* = 100 μm. **E,** Percentage of TUNEL$^+$ cells in the area inside of the vein graft internal elastic lamina 7 days postoperation. **F,** Percentage of TUNEL$^+$ cells in the media and intima of vein grafts 28 days postoperation. The *error bars* indicate the standard error of the mean (day 7, n = 3; day 28, n = 5). *N.S.,* Not significant.

regard to cell origin. Also, siRNA was retained at the application site 7 days postoperation, as found in a study using fluorescence-labelled siRNA.[20]

We confirmed that Girdin expression was suppressed for at least 14 days using atelocollagen-mediated siRNA targeting *Girdin*. We also showed that Girdin expression peaked at ~14 days after bypass grafting and that treatment with atelocollagen-mediated siRNA targeting *Girdin* significantly inhibited intimal hyperplasia in vein grafts 28 days postoperation. Conversely, Girdin depletion did not affect medial thickness 28 days postoperation (Supplementary Fig 3, *B*, online only), although neointimal SMCs may be derived from the media without enhanced proliferation of medial SMCs (Fig 6, *C*). The mechanism by which *Girdin* siRNA inhibits intimal hyperplasia remains unclear. Notably, Girdin depletion did not affect re-endothelialization in vein grafts, which is an important response consideration in bypass grafting.[22,40] Our study indicates that suppressing Girdin in vein grafts could be a useful strategy for preventing vascular stenosis.

CONCLUSIONS

Girdin is pivotal to migration and proliferation of venous SMCs isolated from the neointima of rabbit vein grafts. We confirmed that the combination of atelocollagen and siRNA to allow controlled siRNA release could be a novel therapeutic strategy for vein graft failure and that

Girdin is a potential molecular target for prevention of intimal hyperplasia in vein grafts in vivo.

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AUTHOR CONTRIBUTIONS

Conception and design: HM, SM, AE, YM, KK, MT
Analysis and interpretation: HM, SM, YM, TK, MT
Data collection: HM
Writing the article: HM, SM, MT
Critical revision of the article: HM, SM, AE, YM, TK, NA, KK, MT
Final approval of the article: HM, SM, AE, YM, TK, NA, KK, MT
Statistical analysis: HM, SM
Obtained funding: KK, MT
Overall responsibility: MT

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SUPPLEMENTARY METHODS (online only)

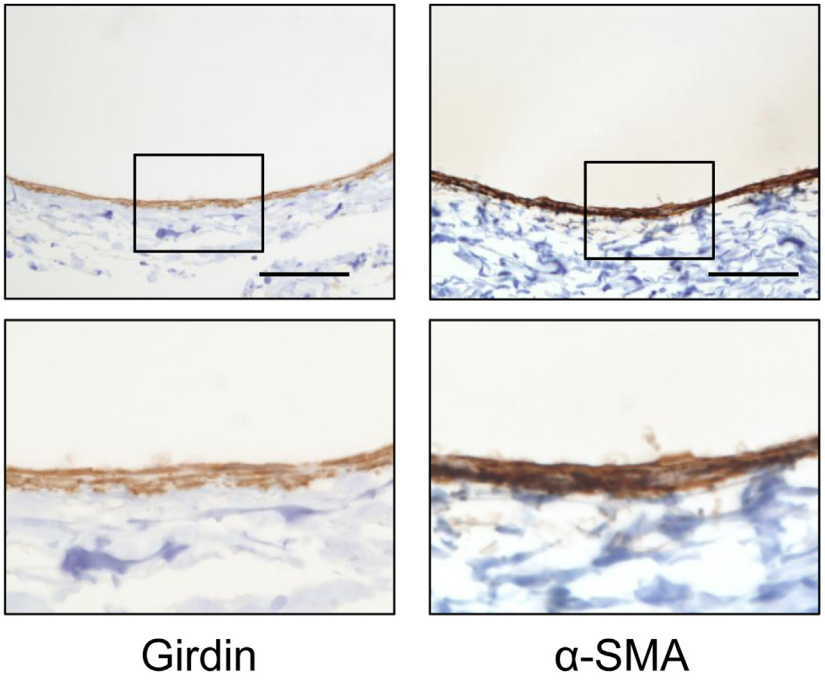
Western blot analysis. After sodium dodecyl sulfate-electrophoresis, proteins were transferred to polyvinylidene fluoride membranes (Millipore Corp, Bedford, Mass). Membranes were blocked in 5% skim milk in Tris-buffered saline with Tween buffer (20-mM Tris-HCl [pH 7.6], 137-mM NaCl, 0.1% Tween 20), with gentle agitation, and incubated with the primary antibody. The membranes were washed with Tris-buffered saline with Tween buffer and incubated with secondary antibody conjugated to horseradish peroxidase. After the membranes were washed, the reaction was visualized by the ECL Detection Kit (GE Healthcare, Buckinghamshire, United Kingdom), according to manufacturer's instructions.

Immunofluorescence staining. Venous smooth muscle cells (SMCs) were plated on culture slides. Cells were fixed with methanol and 4% paraformaldehyde, blocked with 3% bovine serum albumin in phosphate-buffered saline, and stained with anti-β-actin antibody and the rabbit polyclonal anti-Girdin antibody overnight at 4°C. They were then incubated with Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin G (IgG) antibody and Alexa Fluor 594-conjugated goat anti-rabbit IgG antibody (Invitrogen, Carlsbad, Calif) for 1 hour. Fluorescence was examined using confocal laser-scanning microscopy (Fluoview FV500; Olympus, Tokyo, Japan).

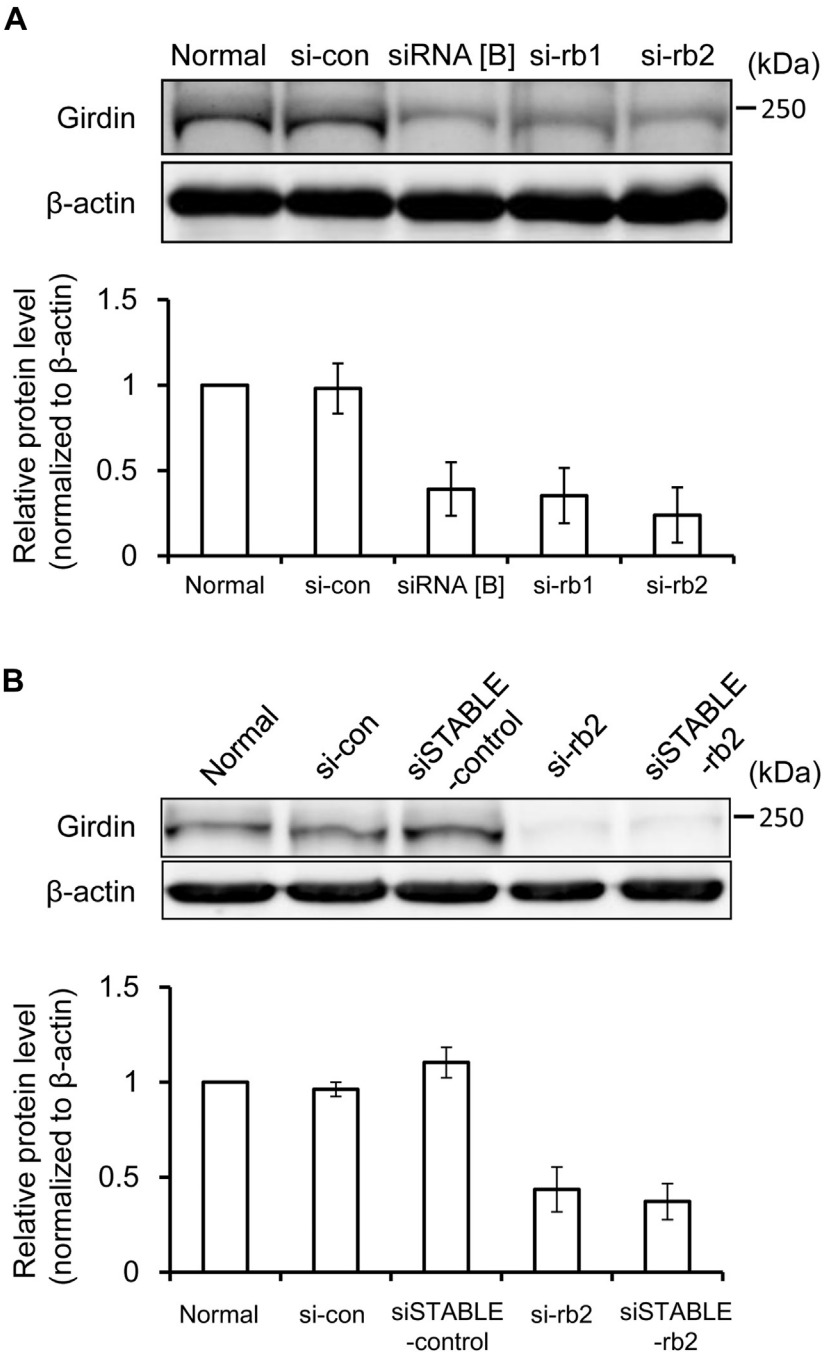
In rabbit vein grafts, transverse sections (4 μm) were blocked with Protein Block Serum-Free Ready-to-Use (Dako, Glostrup, Denmark) and incubated with a mouse monoclonal anti-α-smooth muscle actin antibody and sheep polyclonal anti-Girdin antibody overnight at 4°C. Thereafter, the sections were incubated with donkey anti-mouse IgG antibody conjugated with Alexa Fluor 488 and anti-sheep IgG antibody conjugated with Alexa Fluor 594. 4′,6-Diamidino-2-phenylindole was used as counterstain.

Cell migration assay. Venous SMCs were seeded on 35-mm glass-bottom dishes and transfected with control or *Girdin* small interfering RNA. Confluent cells were scratched with a 200-μL disposable plastic pipette tip and allowed to migrate toward the wound. Wounded cells were incubated with 10% fetal bovine serum in Dulbecco's Modified Eagle's Medium at 37°C, and digital images were taken with an IX70 microscope (Olympus). Wound closure rates were calculated as percentages of migratory distances of control cells (100%).

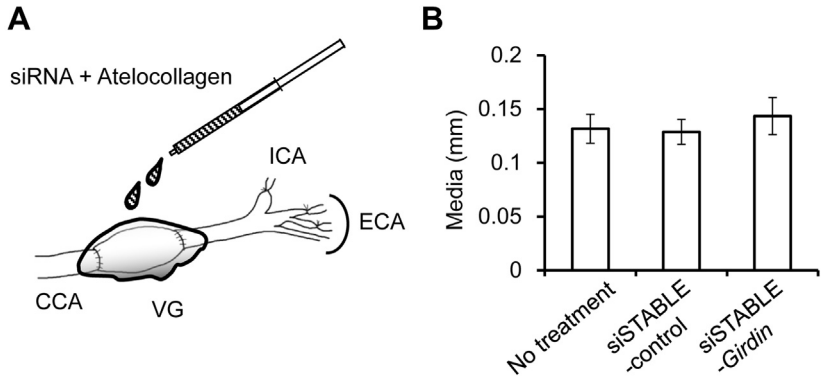
Mitogenic assay. Venous SMCs (2.0×10^4 cells/well) transfected with control or *Girdin* small interfering RNA were plated in 96-well plates 48 hours after transfection and allowed to proliferate for 2 or 4 days with Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum. Cell Proliferation Reagent WST-1 assay (Roche Applied Science, Penzberg, Germany) reagent (10 μL) was added to 100 μL of cell suspension and incubated for 4 hours. The absorbance of each well was measured at 450 nm using a microplate reader.



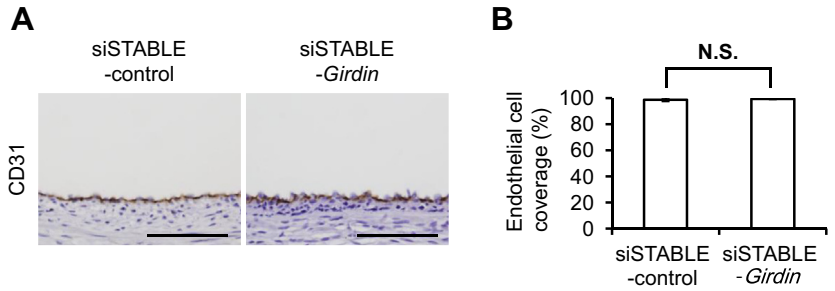
Supplementary Fig 1 (online only). Girdin localizes to venous smooth muscle cells (SMCs) in the vessel walls of intact veins. **Upper panels,** Rabbit intact veins were assessed with immunohistochemical analysis using anti-Girdin and anti-α-smooth muscle actin (*SMA*) antibodies. Note that these veins were dilated with infusion pressure during fixation, and vascular walls look thinner than under normal conditions. **Lower panels,** The *boxed areas* are magnified. The *scale bar* = 100 μm.



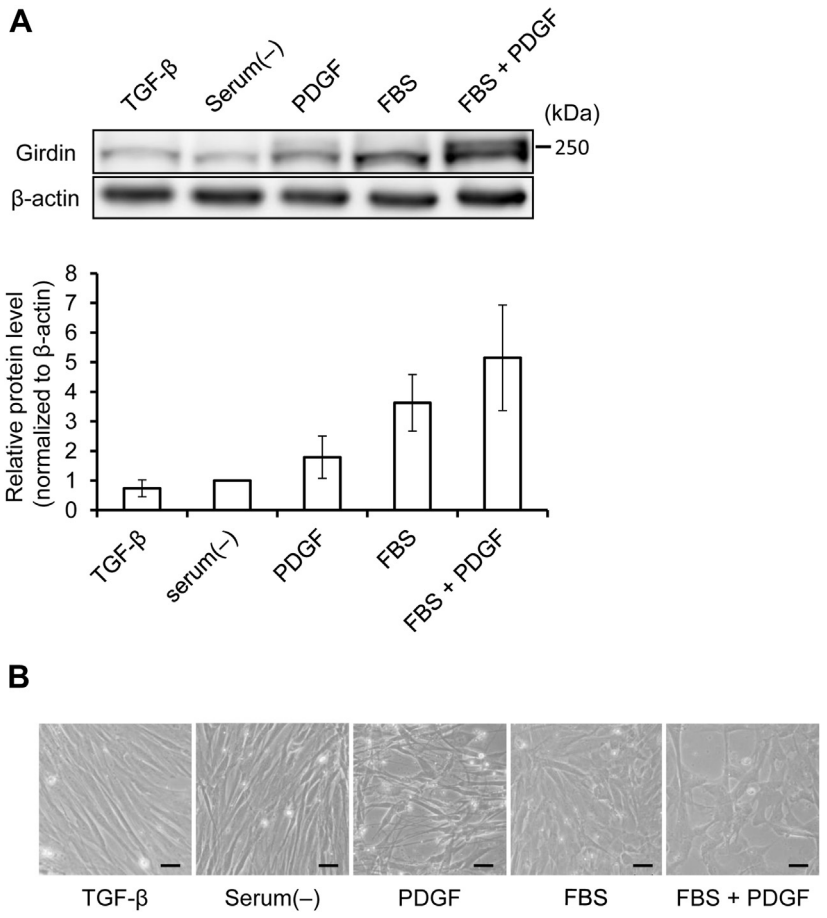
Supplementary Fig 2 (online only). Effects of small interfering RNA (*siRNA*) on Girdin expression in cultured cells. **A,** Venous smooth muscle cells (SMCs) were transfected with three types of Girdin siRNAs or control siRNA, or were not treated (*normal*). A representative Western blot for Girdin is shown. Relative densitometric intensities of Western blot bands for Girdin are presented as means ± standard error of the means in the graph (n = 3). **B,** Stabilized siRNA (*siSTABLE*) retained inhibitory effects equal to those of unmodified siRNA (n = 3).



Supplementary Fig 3 (online only). Local application of atelocollagen-mediated small interfering RNA (siRNA). **A,** siRNA mixed with atelocollagen was used to coat external surfaces of the vein graft (*VG*). *CCA*, Common carotid artery; *ECA*, external carotid artery; *ICA*, internal carotid artery. **B,** There were no significant differences in medial thickness among vein grafts nontreated and treated with Girdin siRNA or control siRNA at 4 weeks postoperation. The *error bars* indicate the standard error of the mean (n = 5 for each group).



Supplementary Fig 4 (online only). Immunohistochemical analysis of vein grafts using anti-CD31 antibody. **A,** Representative immunostaining with anti-CD31 antibody. The *scale bar* = 100 μm. **B,** Percentage of luminal coverage by endothelial cells. The *error bars* indicate the standard error of the mean (n = 3 per each group). *N.S.*, Not significant.



Supplementary Fig 5 (online only). Girdin expression level in phenotypic modulation in venous smooth muscle cells (SMCs). **A,** Western blot analyses show Girdin protein levels in venous SMCs treated with transforming growth factor-β (*TGF-β*; 2.5 μg/L), platelet-derived growth factor (*PDGF*)-BB (25 μg/L), 10% fetal bovine serum (*FBS*), or both FBS and PDGF-BB for 48 hours. Relative densitometric intensities of Western blot bands of Girdin are presented as means ± standard error of the mean in the graph. Girdin protein level in serum-starved venous SMCs [*serum(−)*] was defined as 1 (n = 3). **B,** Morphologic changes in venous SMCs with indicated stimulation for 48 hours. The *scale bar* = 60 μm.